Tuning Escherichia coli for membrane protein overexpression

Wagner, Samuel; Klepsch, Mirjam M.; Schlegel, Susan; Appel, Ansgar; Draheim, Roger; Tarry, Michael; Hogbom, Martin; van Wijk, Klaas J.; Slotboom, Dirk J.; Persson, Jan O.

Published in:
Proceedings of the National Academy of Sciences of the United States of America

DOI:
10.1073/pnas.0804090105

Publication date:
2008

Tuning *Escherichia coli* for membrane protein overexpression

Samuel Wagner*†‡, Mirjam M. Klepsch*, Susan Schlegel*, Ansgar Appel*, Roger Draheim*, Michael Tarry*, Martin Högbom*, Klaas J. van Wijk§, Dirk J. Slotboom¶, Jan O. Persson‖, and Jan-Willem de Gier*†**

*Center for Biomembrane Research, Department of Biochemistry and Biophysics, ‖Department of Mathematics and Statistics, and †Xbrane Bioscience AB, Arrhenius Laboratories, Stockholm University, SE-106 91 Stockholm, Sweden; §Department of Plant Biology, Cornell University, Ithaca, NY 14853; and ¶Department of Biochemistry, University of Groningen, Nyenborg 4, 9747 AG Groningen, The Netherlands

Edited by Douglas C. Rees, California Institute of Technology, Pasadena, CA, and approved July 30, 2008 (received for review April 28, 2008)

A simple generic method for optimizing membrane protein overexpression in *Escherichia coli* is still lacking. We have studied the physiological response of the widely used ''Walker strains'' C41(DE3) and C43(DE3), which are derived from BL21(DE3), to membrane protein overexpression. For unknown reasons, overexpression of many membrane proteins in these strains is hardly toxic, often resulting in high overexpression yields. By using a combination of physiological, proteomic, and genetic techniques we have shown that mutations in the *lac*UV5 promoter governing expression of T7 RNA polymerase are key to the improved membrane protein overexpression characteristics of the Walker strains. Based on this observation, we have engineered a derivative strain of *E. coli* BL21(DE3), termed Lemo21(DE3), in which the activity of the T7 RNA polymerase can be precisely controlled by its natural inhibitor T7 lysozyme (T7Lys). Lemo21(DE3) is tunable for membrane protein overexpression and conveniently allows optimizing overexpression of any given membrane protein by using only a single strain rather than a multitude of different strains. The generality and simplicity of our approach make it ideal for high-throughput applications.

engineering | systems biotechnology | proteomics

The natural abundance of membrane proteins is typically too low to isolate sufficient amounts of material for functional and structural studies. Therefore, membrane proteins must be obtained by overexpression, and the bacterium *E. coli* is the most widely used vehicle for this purpose (1). Although many membrane proteins can be overexpressed in inclusion bodies, their refolding into functional proteins is often not successful (2). To avoid the refolding problem, overexpression of membrane proteins by accumulation in the cytoplasmic membrane is needed. However, overexpression is often toxic to the cell, thereby preventing biomass formation and severely reducing yields (1). Thus, membrane protein overexpression has to be optimized, but no systematic, generic, and high-throughput-compatible method is available for the optimization process.

Bacteriophage T7 RNA polymerase (T7RNAP) is often used to drive recombinant protein production in *E. coli* (3). In BL21(DE3) and its derivatives, the gene encoding T7RNAP is under control of the *lac*UV5 promoter, a strong variant of the wild-type *lac* promoter. It is insensitive to catabolite repression and, therefore, controlled only by the *lac* repressor, LacI, which binds to the *lac* operator (4). T7RNAP exclusively recognizes the T7 promoter and it transcribes eight times faster than *E. coli* RNAP allowing high yield protein production (5). Most T7 expression vectors employ a T7*lac* hybrid promoter that combines the strong T7 ϕ10 promoter with a *lac* operator to diminish leaky expression. On addition of the inducer isopropyl β-D-thiogalactoside (IPTG), *lacI* repression is relieved, resulting in recombinant protein production. If toxicity due to leaky expression is a problem, T7RNAP activity can be further dampened with the T7RNAP inhibitor T7Lys (6). For T7Lys expression, usually the pLysS and pLysE plasmids are used; pLysS hosts produce low amounts of T7Lys, whereas pLysE hosts produce much more enzyme and, therefore, provide a more stringent control (6).

Recently, we studied the physiological response of *E. coli* BL21(DE3)pLysS to membrane protein overexpression (7). Our aim was to identify potential bottlenecks that hamper membrane protein overexpression and to use this information to engineer strains with improved overexpression characteristics. We found that membrane protein overexpression resulted in accumulation of cytoplasmic aggregates containing the overexpressed protein as well as chaperones, proteases, many essential cytoplasmic proteins, and many precursors of periplasmic and outer membrane proteins. Also, levels of respiratory chain complexes in the cytoplasmic membrane were strongly reduced, causing the induction of the AcO-pta pathway for ATP production and downregulation of the tricarboxylic acid (TCA) cycle, resulting in inefficient ATP production. Presumably, these effects were caused by saturation of the Sec translocon, which mediates both translocation of secretory proteins across and integration of membrane proteins into the cytoplasmic membrane.

As a complementary approach, we decided to characterize the so-called Walker strains C41(DE3) and C43(DE3). These strains were selected almost a decade ago in a screen that was designed to isolate derivatives of BL21(DE3) with improved membrane protein overexpression characteristics (8). Overexpression of many membrane proteins in these strains is hardly toxic, often resulting in high overexpression yields (8). The reason for their improved membrane protein overexpression characteristics is not understood.

Here, we report an in-depth characterization of the Walker strains by using a combination of 1D- and 2D-gel electrophoresis and mass spectrometry, complemented with Western blotting, enzymatic activity assays, flow cytometry, and genetics. This analysis has allowed us to identify the key mutations behind the improved membrane overexpression characteristics. Based on this previously undescribed insight, we have engineered an *E. coli* strain that is tunable for membrane protein overexpression and conveniently allows optimizing overexpression of any given protein by using only a single strain rather than a multitude of different strains.

Author contributions: S.W., M.M.K., S.S., D.J.S., J.O.P., and J.-W.d.G. designed research; S.W., M.M.K., S.S., A.A., D.J.S., and J.O.P. performed research; R.D., M.T., and M.H. contributed new reagents/analytic tools; S.W., M.M.K., S.S., A.A., K.J.v.W., D.J.S., J.O.P., and J.-W.d.G. analyzed data; and S.W., K.J.v.W., and J.-W.d.G. wrote the paper.

Conflict of interest statement: S.W. and J.-W.d.G. are cofounders of the biotech company Xbrane Bioscience AB.

This article is a PNAS Direct Submission.

‡Present address: Section of Microbial Pathogenesis, Boyer Center for Molecular Medicine, Yale School of Medicine, New Haven, CT 06536.

**To whom correspondence should be addressed. E-mail: degier@dbb.su.se.

This article contains supporting information online at www.pnas.org/cgi/content/full/0804090105/DCSupplemental.

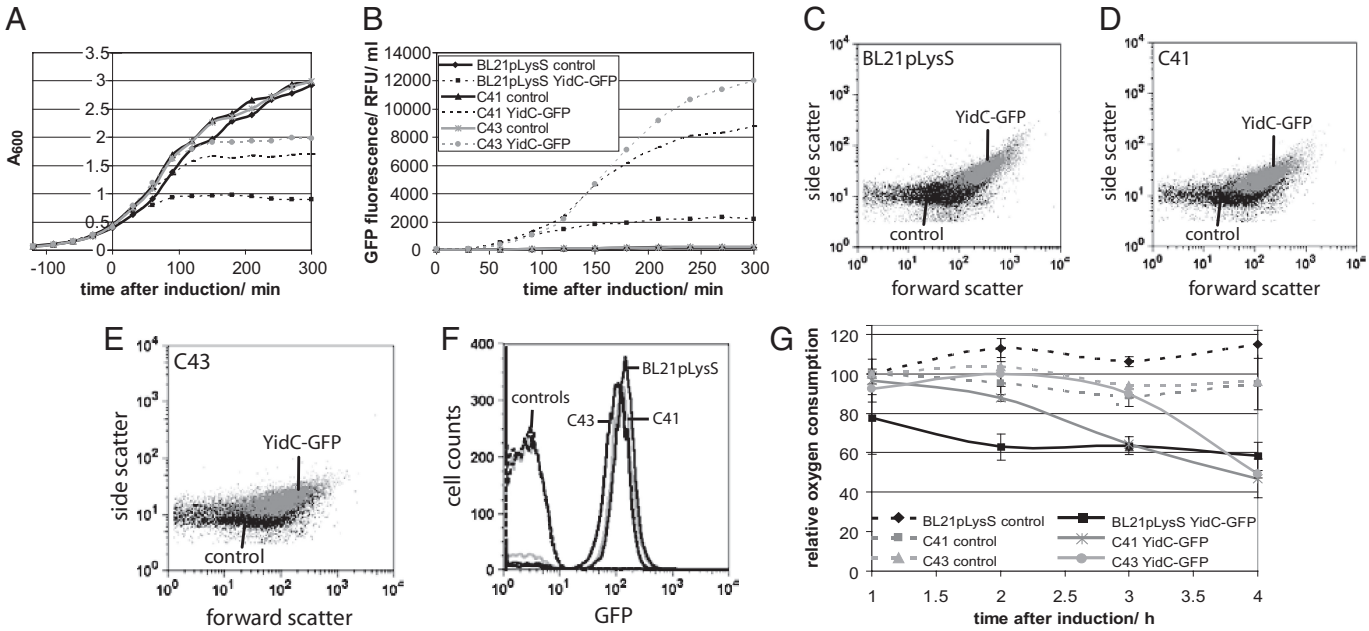

Fig. 1. Analysis of growth, protein expression, morphology, and respiration of BL21(DE3)pLysS, C41(DE3), and C43(DE3) overexpressing YidC-GFP. (*A* and *B*) Growth (*A*) and protein expression of cells overexpressing YidC-GFP (*B*) were monitored by measuring the A_{600} and GFP fluorescence, respectively, every 30 min. (*C–F*) The following parameters were monitored by flow cytometry: forward and side scatter (*C–E*), which provide information about cell size and granularity, and GFP fusion protein expression (*F*). For *C–F*, cells were harvested 4 h after induction with IPTG. (*G*) Oxygen consumption was measured in whole cells every hour. Experiments were done in triplicate. Respiratory activities of control cells were set to 100.

Results

To compare the consequences of membrane protein overexpression in C41(DE3), C43(DE3), and BL21(DE3)pLysS, we used the well characterized YidC-GFP fusion as the initial test protein (7). In this fusion, the membrane protein YidC is C-terminally fused to GFP. YidC-GFP is predominantly overexpressed in the membrane and its GFP-moiety allows convenient monitoring of overexpression levels by using fluorescence detection (9, 10).

Growth, Overexpression, and Morphology. YidC-GFP overexpression affected growth of both C41(DE3) and C43(DE3) much less than of BL21(DE3)pLysS (Fig. 1*A*). After 6-h induction, yields were 4- and 6-fold higher in cultures of C41(DE3) and C43(DE3), compared with the BL21(DE3)pLysS culture (Fig. 1*B*). Flow cytometry showed that on YidC-GFP induction both the forward scatter and side scatter increased less in C41(DE3) and C43(DE3), compared with BL21(DE3)pLysS (Fig. 1 *C–E*), indicating that morphology and cell division of the Walker strains are less affected by membrane protein overexpression compared with BL21(DE3)pLysS. However, flow cytometry showed that YidC-GFP expression per cell was similar for all three strains (Fig. 1*F*).

Effects of Membrane Protein Overexpression on The C41(DE3) and C43(DE3) Proteomes. The subproteomes of C41(DE3), C43(DE3), and BL21(DE3)pLysS were analyzed by 1D- and 2D-gel electrophoresis and mass spectrometry, complemented with Western blotting and enzymatic activity assays. There were only minor differences between the subproteomes of cells not overexpressing YidC-GFP [supporting information (SI) Figs. S1, S2*A*, S3, and S4, Table S1, and Table S2].

Overexpression of YidC-GFP in BL21(DE3)pLysS strongly affected complexes in the membrane involved in respiration, presumably leading to the inefficient production of ATP by means of the AcO-pta pathway (7). The better growth of C41(DE3) and C43(DE3) on membrane protein overexpression compared with BL21(DE3)pLysS could be the result of more efficient respiration due to a less severe perturbation of the cytoplasmic membrane proteome. Surprisingly, after 4 h of YidC-GFP overexpression, the cytoplasmic membrane proteomes of C41(DE3) and C43(DE3) were affected in a similar way as in BL21(DE3)pLysS (Figs. S1 and S2*A* and Table S1). This observation was corroborated by enzymatic activity assays of some key complexes involved in energy transduction (Fig. S2*B*). However, when oxygen consumption was measured over time it became clear that on induction of YidC-GFP overexpression the decrease in oxygen consumption was instantaneous in BL21(DE3)pLysS, but only gradual in C41(DE3) and C43(DE3) (Fig. 1*G*).

The accumulation levels of the chaperones ClpB and IbpA, and the protease HslUV, all involved in resolving cytoplasmic aggregates, were much lower in C41(DE3) and C43(DE3) than in BL21(DE3)pLysS (Fig. 2 *A* and *B*). This observation, together with the flow cytometry data, suggests that C41(DE3) and C43(DE3) do not suffer from cytosolic protein misfolding and aggregation as observed in BL21(DE3)pLysS (7). Indeed, hardly any protein aggregates could be isolated from the Walker strains (Fig. 2*C*).

The effect of membrane protein overexpression on the accumulation levels of the processed forms of most secretory proteins in C41(DE3) and C43(DE3) was only marginal compared with BL21(DE3)pLysS (Fig. S4*B*). This result is in keeping with the observation that the aggregates isolated from BL21(DE3)pLysS contain many precursors of secretory proteins (7). No differences in accumulation levels of key components involved in membrane protein targeting and the Sec translocon were observed between the three strains (data not shown).

Taken together, the analysis of the subproteomes of C41(DE3), C43(DE3), and BL21(DE3)pLysS and the oxygen consumption measurements indicate that on membrane protein overexpression, C41(DE3) and C43(DE3) experience less of a Sec translocon saturation problem than BL21(DE3)pLysS. We hypothesized that this effect could be due to differences in membrane protein overexpression kinetics.

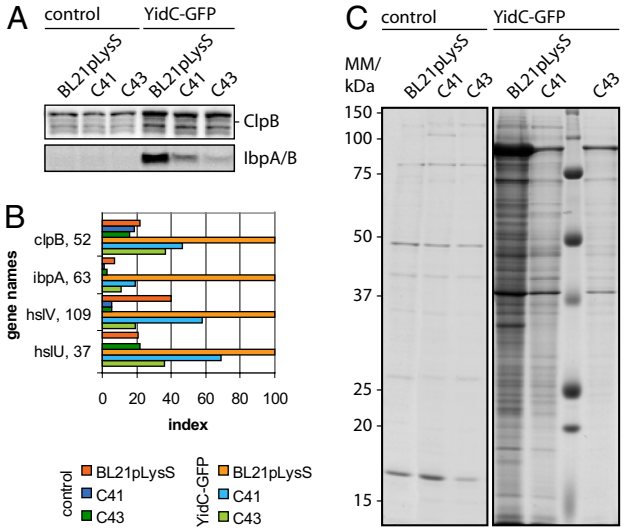

Fig. 2. Analysis of subproteomes of BL21(DE3)pLysS, C41(DE3), and C43(DE3) overexpressing YidC-GFP. (*A*) Proteins of whole cell lysates of cells overexpressing YidC-GFP fusions for 4 h were separated by means of SDS/PAGE and subsequently subjected to Western blotting with antibodies to ClpB and IbpA/B. (*B*) Proteins of whole cell lysates of cells overexpressing YidC-GFP fusions for 4 h were separated by means of 2D IEF/SDS/PAGE. Shown is the relative quantification of protein spots representing ClpB, IbpA, HslV, and HslU. (*C*) Protein aggregates were isolated from cells overexpressing YidC-GFP fusions as described in the *Materials and Methods* section. The aggregates were analyzed by 1D SDS/PAGE, and gels were stained with colloidal Coomassie.

Membrane Protein Overexpression Kinetics. To monitor YidC-GFP overexpression kinetics on-line, we cultured cells in a 96-well plate in a spectrofluorometer. Expression started at a higher rate in BL21(DE3)pLysS than in C41(DE3) and C43(DE3) and remained constant (Fig. 3*A*). However, the expression rates of C41(DE3) and C43(DE3) were low initially but increased over time, and after 2 h the C41(DE3) and C43(DE3) strains even outperformed BL21(DE3)pLysS. The lower initial expression rate in C41(DE3) and C43(DE3) is most likely due to lower transcript levels of the overexpressed protein. Indeed, real-time (RT) PCR showed that YidC-GFP mRNA levels in C41(DE3) and C43(DE3) were lower than in BL21(DE3)pLysS and that the onset of transcription was slightly more delayed in C43(DE3) than C41(DE3) (Fig. 3*B*). This result is in agreement with the observation that transcript levels of overexpressed proteins in the C41(DE3) and C43(DE3) strains are lower than in BL21(DE3) (8).

Why are the transcript levels lower in the Walker strains? Mutations in the gene encoding T7RNAP could make the enzyme less active (11). However, there were no mutations in the T7RNAP gene sequences in the Walker strains (data not shown). To test whether the weaker expression was due to a slower uptake of IPTG, the accumulation levels of the IPTG translocator lactose permease (LacY) in the membrane were determined. LacY expression is induced on addition of IPTG. In nonoverexpressing cells, LacY accumulation kinetics of BL21(DE3)pLysS and C41(DE3) were comparable, whereas the onset of LacY expression was slightly delayed in C43(DE3) (Fig. 3*C*). This delay may explain the minute difference between C41(DE3) and C43(DE3) observed during the on-line fluorescence measurements (Fig. 3*A*), but does not explain the divergence between the Walker strains and BL21(DE3)pLysS.

Next, we monitored the accumulation levels of T7RNAP by Western blotting. Whereas T7RNAP was visible already 30 min after induction with IPTG and reached maximum intensity after 90 min in BL21(DE3)pLysS, only low levels of T7RNAP could be detected in C41(DE3) and C43(DE3), even after 120-min induction (Fig. 3*C*). mRNA transcript levels of T7RNAP corroborated the Western blotting experiments (Fig. 3*D*). This difference raised the question whether the *lac*UV5 promoter that mediates T7RNAP expression is mutated in the Walker strains. Indeed, sequencing of the *lac*UV5 promoter revealed that three mutations in the promoters of both C41(DE3) and C43(DE3) had occurred, two in the −10 region of the promoter and one in the *lac* operator just upstream of the symmetric part of the *lac* repressor binding site (Fig. 3*E*). The two mutations in the −10 region revert the *lac*UV5 promoter back into the much weaker wild-type *lac* promoter (4). In contrast to the *lac*UV5 promoter, the wild-type *lac* promoter is susceptible to catabolite repression and requires activation by catabolite repressor protein-cAMP (12, 13). Indeed, the strong catabolite repressor glucose delayed the onset of YidC-GFP expression even more in the Walker strains, but not in BL21(DE3)pLysS (data not shown).

Can The Mutations in The *lac*UV5 Rromoter Be Mimicked by Dampening T7RNAP Activity? How important are the mutations in the *lac*UV5 promoter for the improved membrane protein overexpression characteristics of the Walker strains? We reasoned that dampening T7RNAP activity with the T7RNAP inhibitor T7Lys could mimic this effect and, thus, answer this question. T7Lys was placed under control of an L-rhamnose inducible promoter (*rha*BAD) on a pACYC derived plasmid designated pLemo (Fig. 4*A* and Fig. S5). The *rha*BAD promoter has some features that make it extremely well suited for expression of the T7RNAP inhibitor T7Lys; it is exceptionally well titratable, covers a broad window of expression intensities, and it functions independent of strain background (14). BL21(DE3) transformed with pLemo is hereafter referred to as Lemo21(DE3). Lemo21(DE3) was used to overexpress YidC-GFP. Supplementing cultures with different amounts of L-rhamnose resulted in different and highly reproducible expression levels of YidC-GFP (Fig. 4*B*). There was a clear effect of the L-rhamnose concentration (i.e., level of T7Lys) on the YidC-GFP overexpression and mRNA levels (Fig. 4 *B* and *C*). At an L-rhamnose concentration of 1,000 μM there was a YidC-GFP overexpression optimum in Lemo21(DE3) that was higher than in the Walker strains. Notably, dampening of T7RNAP activity in the Walker strains led, besides a slight increase in Lemo41(DE3) [C41(DE3) with pLemo] at 0-μM L-rhamnose, to decreased expression of YidC-GFP (Fig. 4*B*), indicating that the effects of the promoter mutations and the lowering of T7RNAP activity by T7Lys converge at decreasing recombinant mRNA levels. Taken together, these observations demonstrate that the mutations in the *lac*UV5 promoters in the Walker strains can be mimicked in BL21(DE3) by dampening T7RNAP activity.

Characterization of Lemo21(DE3). Lemo21(DE3) overexpressing YidC-GFP at different L-rhamnose concentrations was characterized in more detail. There was a clear correlation between the L-rhamnose concentration, growth, and protein production (Fig. 5 *A* and *B*). Flow cytometry showed that increasing L-rhamnose concentrations prevented overgrowth of the culture by nonexpressing cells (Fig. 5*C*). Also, aggregate formation was prevented with increasing L-rhamnose concentrations (Fig. S6 *A* and *B*). These observations are in keeping with the only mild induction of ClpB and IbpA/B chaperones in cells overexpressing YidC-GFP at the optimal concentration of L-rhamnose (Fig. S6 *C* and *D*). Last, oxygen consumption was not affected in Lemo21(DE3) at the optimal L-rhamnose concentration (Fig. 5*D*).

Closing The Circle with a Promoter Swap. All experiments with Lemo21(DE3) indicated that the mutations in the *lac*UV5 promoters of the Walker strains are key to their improved

BIOCHEMISTRY

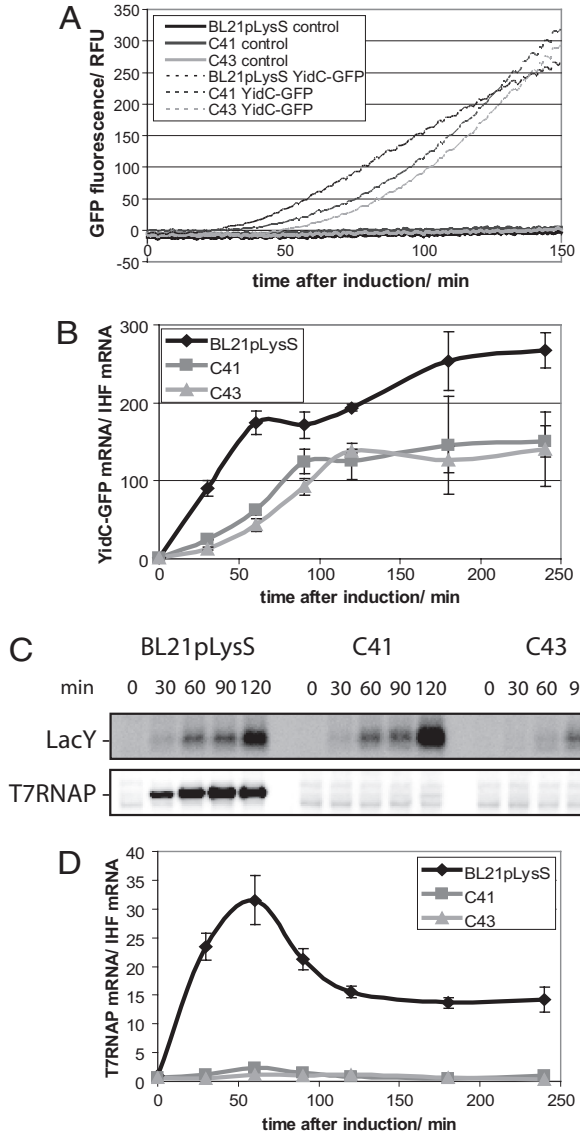

Fig. 3. Mutations in the *lac*UV5 promoter controlling T7RNAP transcription lead to reduced T7RNAP expression levels in C41(DE3) and C43(DE3). (*A*) YidC-GFP overexpression was monitored on-line by measuring GFP fluorescence every 30 sec in cells cultured in a 96-well plate in a spectrofluorometer. (*B*) Quantification of mRNA levels of YidC-GFP by RT PCR. Cells overexpressing YidC-GFP were harvested at indicated timepoints after induction of expression with IPTG. Subsequently, mRNA was isolated and cDNA transcribed as described in the *Materials and Methods* section. Experiments were done in triplicate. (*C*) Accumulation levels of LacY in the membrane and T7RNAP in whole cells over time as monitored by Western blotting. (*D*) Quantification of mRNA levels of T7 RNA polymerase was done by RT PCR. Experiments were done in triplicate. (*E*) Sequences of the promoters controlling transcription of T7 RNA polymerase in BL21(DE3), C41(DE3), and C43(DE3) were determined as described in *Materials and Methods*. Mutations are shaded gray.

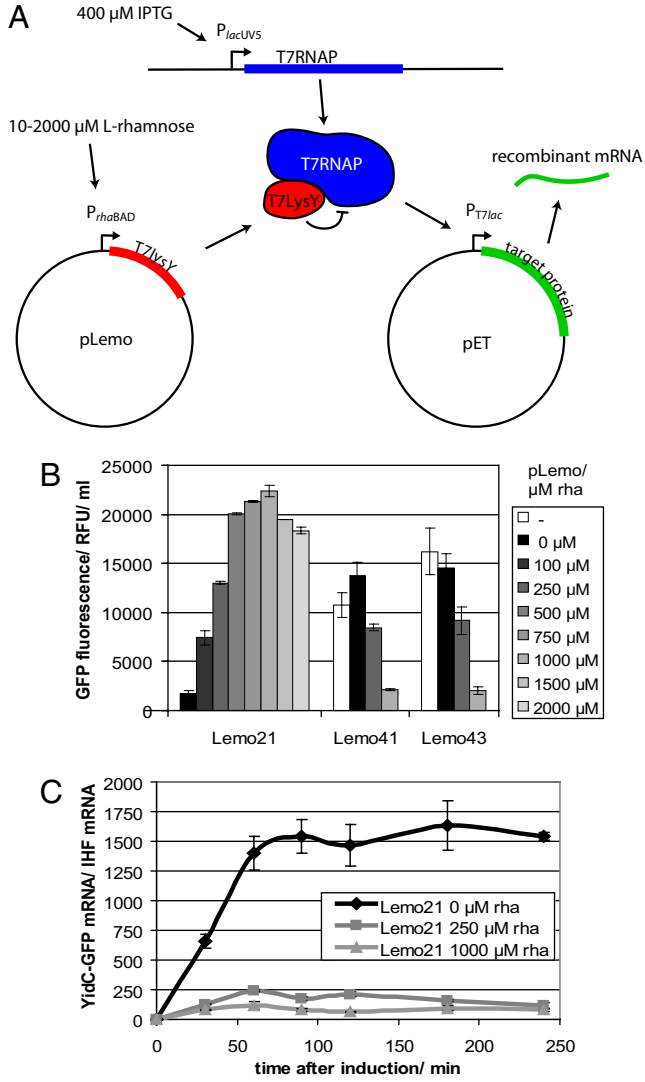

Fig. 4. Tuning T7 RNA polymerase activity with T7Lys. (*A*) Model of tuning T7 RNA polymerase activity with T7Lys. Expression of T7Lys is under the control of the *rhaBAD* promoter and is induced by the addition of L-rhamnose. The amidase activity deficient T7Lys mutant LysY was used (Fig. S5). (*B*) Expression of YidC-GFP in Lemo21(DE3), Lemo41(DE3) [C41(DE3) with pLemo], and Lemo43(DE3) [C43(DE3) with pLemo] at different L-rhamnose concentrations. C41(DE3) and C43(DE3) were included as controls (white bars). Experiments were done in triplicate. (*C*) Cells overexpressing YidC-GFP were harvested at indicated timepoints after induction with IPTG and mRNA levels of YidC-GFP were determined by RT PCR. Experiments were done in triplicate.

membrane protein overexpression characteristics. To put this hypothesis to the test directly, we swapped the *lac* promoters between BL21(DE3) and C43(DE3). Expression of YidC-GFP in the absence and presence of pLemo, and levels of T7RNAP, ClpB, and IbpA/B in the strains with swapped promoters showed that BL21(DE3) could be converted into a Walker strain and vice versa (Fig. 6 *A* and *B*).

An "All in One" Platform for Membrane Protein Overexpression. To assess the generality of Lemo21(DE3), expression of 15 membrane protein-GFP fusions (11 *E. coli*, one *Shewanella oneidensis*, one rat, and two human membrane proteins) was tested in BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE, C41(DE3), C43(DE3), and Lemo21(DE3). Expression of soluble GFP was also tested. First, the optimal concentration of L-rhamnose, from

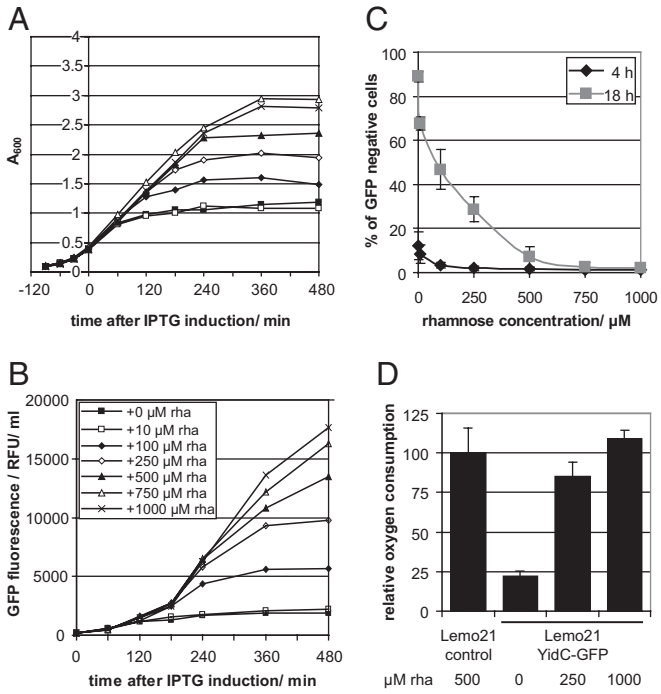

Fig. 5. Characterization of Lemo21(DE3). In all experiments, Lemo21(DE3) overexpressed YidC-GFP in the presence of the indicated amounts of L-rhamnose. (*A–C*) Growth (*A*) and protein expression of Lemo21(DE3) (*B*) were monitored by measuring the A_{600} and GFP fluorescence, respectively, every 1–2 h. Fraction of cells not expressing YidC-GFP 4 and 18 h after induction as monitored by flow cytometry (*C*). Experiments were done in triplicate. (*D*) Oxygen consumption was measured in whole cells four hours after induction. Experiments were done in triplicate. Respiratory activities of control cells were set to 100.

0 to 2,000 µM, was determined for each protein in Lemo21(DE3) (data not shown). Next, overexpression in the different strains was compared (Fig. 6*C*). Expression was consistently best 8 h after induction with IPTG. The *E. coli* osmolarity sensor protein EnvZ and the *S. oneidensis* transporter A (SOTA) only expressed well in BL21(DE3) and Lemo21(DE3) without any L-rhamnose. In most cases, Lemo21(DE3) outperformed BL21(DE3) and the pLysS/E variants, and yields were comparable with the ones in C41(DE3) and C43(DE3) or even higher. In general, GFP fluorescence correlates well with functional overexpression levels of membrane proteins (15). This correlation was confirmed here for the glutamate transporter GltP (data not shown). Thus, Lemo21(DE3) conveniently allows optimizing overexpression of membrane proteins by using only one strain and a simple L-rhamnose titration rather than a multitude of different strains.

Discussion

Our recent study on the consequences of membrane protein overexpression in *E. coli* resulted in a plethora of potential leads for engineering strains with improved membrane protein overexpression characteristics (7). To narrow down the number of leads, we have now studied the consequences of membrane protein overexpression in the Walker strains (8). Overexpression of many membrane proteins in these BL21(DE3) derivatives is hardly toxic; i.e., growth is only marginally affected often resulting in high membrane protein overexpression yields. By using a combination of proteomics and genetics, we show that mutations in the promoter governing expression of T7 RNA polymerase are key to the improved membrane protein overexpression characteristics of the Walker strains.

How can such minor modifications have such a large impact on membrane protein overexpression yields? In BL21(DE3), the strong *lac*UV5 promoter is used for T7RNAP expression (16). T7RNAP exclusively recognizes the T7 promoter and transcribes eight times faster than *E. coli* RNAP. The reasoning behind the choice of these components to create a protein overexpression system was straight-forward; more mRNA results in more overexpressed protein (16). However, for most membrane proteins this strong overexpression leads to the production of more protein than the Sec translocon can process. Saturation of the Sec translocon makes it for most overexpressed and endogenous membrane proteins impossible to insert into the membrane. This results in perturbation of the membrane proteome. Membrane proteins that cannot insert into the membrane end up in the

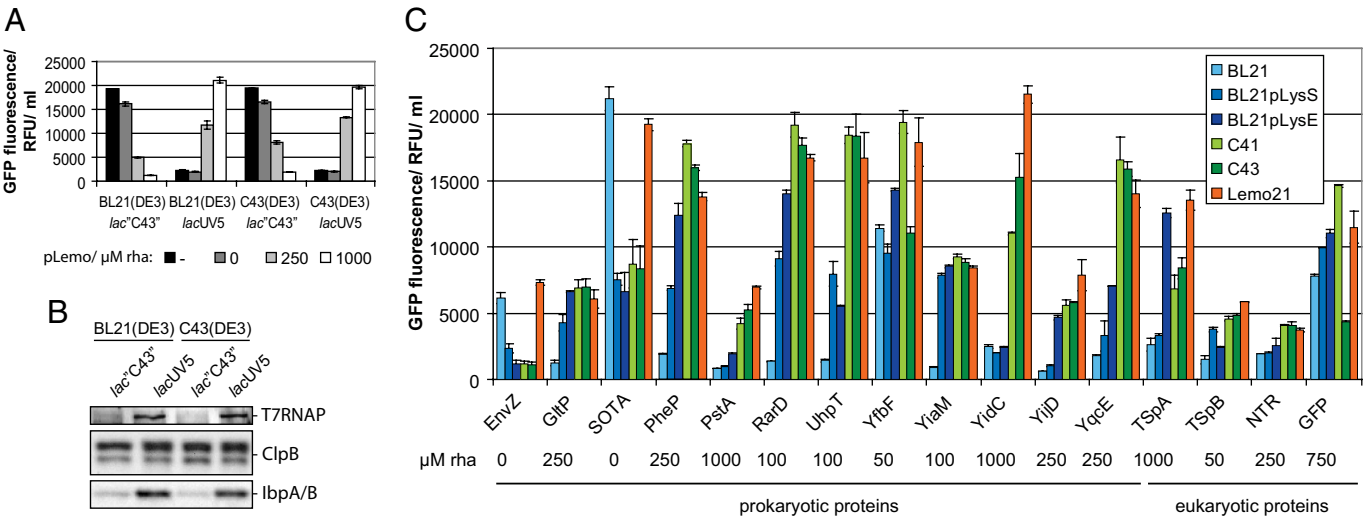

Fig. 6. Converting BL21(DE3) into C43(DE3) and vice versa, and overexpression screen. (*A* and *B*) Promoters controlling T7RNAP expression in BL21(DE3) and C43(DE3) were swapped, and YidC-GFP expression in the strain with swapped promoters in the absence and presence of pLemo was monitored by GFP-fluorescence (*A*), and T7RNAP, ClpB, and IbpA/B accumulation levels were monitored in the strains with swapped promoters and their controls by Western blotting (*B*). (*C*) Overexpression of membrane protein GFP-fusions, and GFP in different strains was monitored by measuring GFP fluorescence 8 h after induction. For graphical reasons, fluorescence values of TSpA and B, and NTR were multiplied by 10 and fluorescence values of GFP were divided by 50.

cytoplasm and may aggregate. Translocation of secretory proteins is also hampered when the Sec translocon is saturated, leading to further protein aggregation. And as an indirect effect of this saturation of the Sec translocon, ATP synthesis becomes inefficient.

Either by the mutations in the *lac*UV5 promoter or by dampening T7RNAP activity with T7Lys, transcription of most genes encoding the recombinant membrane proteins can be tuned such that saturation of the Sec translocon does not occur. Thus, by harmonizing translation and insertion into the membrane of the recombinant membrane protein, the toxic effects of overexpression are minimized. The result is the formation of more biomass that can produce membrane proteins. Notably, the key to improved membrane protein overexpression yields is not an increased amount of overexpressed membrane protein per cell, but increased amounts of biomass. This observation indicates that yields per cell are bound to a maximum possible due to space constraints.

There are minor differences in membrane protein overexpression kinetics between C41(DE3) and its derivative C43(DE3) (8). On IPTG induction, expression of LacY in C43(DE3) is slightly delayed. Consistently, also the expression of T7RNAP and the recombinant protein, and perturbation of the membrane proteome are slightly delayed in C43(DE3) compared with C41(DE3). All of these results are in keeping with the observation that in Lemo41(DE3) background expression of T7Lys leads to somewhat improved expression of YidC-GFP, whereas in Lemo43(DE3), it has a negative effect. Although, the differences in T7RNAP activity between C41(DE3) and C43(DE3) hardly affect membrane protein overexpression, they explain the relatively low expression levels of GFP and other soluble proteins in C43(DE3) (Fig. 6*C*) (8). Transcription kinetics in C43(DE3) do not make it a suitable vehicle for the overexpression of soluble proteins, whereas Lemo21(DE3) can be easily tuned both for soluble and membrane proteins.

In conclusion, the systems biotechnology approach used here to characterize the Walker strains has not only enabled us identify the mutations that are key to their improved membrane overexpression characteristics but has also made it possible for us to engineer an *E. coli* BL21(DE3)-derived strain, Lemo21(DE3), that is tunable for overexpression. This strain conveniently allows optimizing overexpression of both membrane and soluble proteins by using only a single strain and a simple L-rhamnose titration rather than a multitude of different strains and is ideally suited to high-throughput screening procedures.

Materials and Methods

Protein Overexpression and Assessment of Host Cell Physiology. Detailed information about strains, plasmids, and culture conditions used can be found in *SI Materials and Methods*. GFP fluorescence measurements and flow cytometry were performed essentially as described before (7, 9, 10). See *SI Materials and Methods* for details. Oxygen consumption in whole cells was measured as described before (7).

Analyses of Subproteomes. The accumulation levels of ClpB, IbpA/B, and T7RNAP in whole cell lysates and LacY in cytoplasmic membranes were monitored by Western blotting as described before (7). Protein aggregates were isolated and analyzed as described before (7, 17); 2D-gel electrophoresis, image analysis of 2D-gels as well as protein identification by mass spectrometry was performed essentially as described before (7, 18), and statistical analysis of 2D-gels was done by ANOVA (Tables S3–S5). See *SI Materials and Methods* for details.

Genetic Techniques. Standard protocols were used for purification of mRNA, reverse transcription, and quantitative PCR as well as for sequencing of *lac*UV5 promoters and genes encoding T7RNAP. Swaps of the promoters controlling expression of T7RNAP in BL21(DE3) and C43(DE3) were performed by using an approach based on the Red Swap method (19). See *SI Materials and Methods* for details.

ACKNOWLEDGMENTS. We thank Joen Luirink, David Wickström, and Gunnar von Heijne for critically reading the manuscript; Michal Zolkiewski, Ronald Kaback, and Bernd Bukau for antisera; Reinhard Grisshammer, David Drew, and Nien-Jen for plasmids; and Claudia Wagner for assistance with the flow cytometry experiments. This work was supported by Grants from the Swedish Research Council, the Carl Tryggers Stiftelse, the National Institutes of Health, and the EMBO Young Investigators Program (to J.-W.d.G.), and The Swedish Foundation for International Cooperation in Research and Higher Education (STINT) Grant (to J.-W.d.G. and K.J.v.W.). Proteomics infrastructure was supported by the New York State Office of Science and Technology Grand (to K.J.v.W.). The Center for Biomembrane Research is supported by the Swedish Foundation for Strategic Research.

1. Wagner S, Bader ML, Drew D, de Gier JW (2006) Rationalizing membrane protein overexpression. *Trends Biotechnol* 24:364–371.
2. Drew D, Fröderberg L, Baars L, de Gier JW (2003) Assembly and overexpression of membrane proteins in Escherichia coli. *Biochim Biophys Acta* 1610:3–10.
3. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* 185:60–89.
4. Arditti RR, Scaife JG, Beckwith JR (1968) The nature of mutants in the lac promoter region. *J Mol Biol* 38:421–426.
5. Iost I, Guillerez J, Dreyfus M (1992) Bacteriophage T7 RNA polymerase travels far ahead of ribosomes in vivo. *J Bacteriol* 174:619–622.
6. Studier FW (1991) Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J Mol Biol* 219:37–44.
7. Wagner S, *et al* (2007) Consequences of membrane protein overexpression in Escherichia coli. *Mol Cell Proteomics* 6:1527–1550.
8. Miroux B, Walker JE (1996) Over-production of proteins in Escherichia coli: Mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol* 260:289–298.
9. Drew D, Lerch M, Kunji E, Slotboom DJ, de Gier JW (2006) Optimization of membrane protein overexpression and purification using GFP fusions. *Nat Methods* 3:303–313.
10. Drew D, *et al* (2005) A scalable, GFP-based pipeline for membrane protein overexpression screening and purification. *Protein Sci* 14:2011–2017.
11. Makarova OV, Makarov EM, Sousa R, Dreyfus M (1995) Transcribing of Escherichia coli genes with mutant T7 RNA polymerases: Stability of lacZ mRNA inversely correlates with polymerase speed. *Proc Natl Acad Sci USA* 92:12250–12254.
12. Silverstone AE, Arditti RR, Magasanik B (1970) Catabolite-insensitive revertants of lac promoter mutants. *Proc Natl Acad Sci USA* 66:773–779.
13. Kolb A, Busby S, Buc H, Garges S, Adhya S (1993) Transcriptional regulation by cAMP and its receptor protein. *Annu Rev Biochem* 62:749–795.
14. Giacalone MJ, *et al* (2006) Toxic protein expression in Escherichia coli using a rhamnose-based tightly regulated and tunable promoter system. *Biotechniques* 40:355–364.
15. Geertsma ER, Groeneveld M, Slotboom DJ, Poolman B (2008) Quality control of overexpressed membrane proteins. *Proc Natl Acad Sci USA* 105:5722–5727.
16. Studier FW, Moffatt BA (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* 189:113–130.
17. Tomoyasu T, Mogk A, Langen H, Goloubinoff P, Bukau B (2001) Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the Escherichia coli cytosol. *Mol Microbiol* 40:397–413.
18. Baars L, *et al* (2006) Defining the role of the Escherichia coli chaperone SecB using comparative proteomics. *J Biol Chem* 281:10024–10034.
19. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc Natl Acad Sci USA* 97:6640–6645.